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AN ABSTRACT OF THE THESIS OF Mark Baustian for the Master of
Science in Biology presented June 20, 1986.

Title: The Contribution of the Lymph Hearts in Compensation for
Acute Hypovolemic Stress in the Toad Bufo marinus.

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

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Stanley H. Ilman, Chairman

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Philip Withers

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Malcolm Lea

The Marine Toad Bufo marinus was used as a model for an investigation of the rate and volume pumping capacity of the amphibian lymph heart. B. marinus is a widely studied terrestrial toad which would be expected to experience a hypovolemic stress as a consequence of evaporative water loss when it is away from water.

Currently published data on the role of the lymphatic system in amphibians are inadequate and contradictory. Estimates of the rate of formation of lymph and the role of the lymph hearts in returning this fluid to the circulation are not based on actual volume determinations

but rather estimates derived from changes in hematocrit using published values of plasma and blood volume. The lymph hearts are known to be vital to the maintenance of normal fluid compartment physiology and to increase their rate of activity during episodes of hypovolemic stress. Yet, significant redistribution of body fluids following hemorrhage appears to occur in animals without lymph hearts.

In this study, plasma and blood volumes were determined by the dye dilution technique using injected Evan's blue dye to label the plasma. Eight intact and 6 animals with their lymph hearts destroyed were hemorrhaged to 78% and 75% of their initial blood volumes, respectively. Changes in blood volume were measured following the hemorrhage by analysis of Evan's blue washout and hemodilution.

The intact animals completely compensated for the acute stress by 17.5 min following hemorrhage. In that time, recovery to 94% of control blood volume was determined by the Evan's blue technique and to 104% by hematocrit dilution. The corresponding rates of fluid return were 0.21 ml/min and 0.33 ml/min, respectively. Analysis of the extracellular fluid volume label, thiocyanate (SCN^-), which was simultaneously injected, indicates that the compensation resulted from redistribution of the extracellular fluid.

Similar experiments done on animals which had their lymph hearts destroyed by cauterization indicated no capacity to replace lost blood volume. Following hemorrhage to 75% of initial blood volume recovery was to only 77% as determined by Evan's blue washout. Post hemorrhage changes in hematocrit indicated continued depletion of blood volume over the same period. This discrepancy is the result of under estimation of

Evan's blue washout caused by 2 factors, the method of analysis and introduction of label to the lymph pool during the course of the experiment.

Plasma and lymph protein concentration was measured to determine the extent of protein redistribution following lymph heart destruction. No significant change in protein concentration was found in either compartment.

The conclusions of the study are that B. marinus can fully recover from an acute volume stress of 20% within 20 min by mobilizing extra-vascular lymph at the rate of approximately 0.2 to 0.3 ml/min and that functional lymph hearts are necessary to accomplish this response.

THE CONTRIBUTION OF THE LYMPH HEARTS IN
COMPENSATION FOR ACUTE HYPOVOLEMIC
STRESS IN THE TOAD BUFO MARINUS

by

MARK BAUSTIAN

A thesis submitted in partial fulfillment
of the requirements for the degree of

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TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

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INTRODUCTION

Amphibians face potentially severe water balance stress whether in aquatic or aerial situations. Because their skin presents a minimal barrier to water flux, they experience either a diffusional water load in hypoosmotic environments or, when in air, they evaporate water at a rate equal to a free surface of water. Yet, amphibians occupy diverse habitats ranging from totally aquatic to fully terrestrial.

Many unique adaptations are expressed to meet this dichotomous challenge to hydrational homeostasis. Thorson (1955, 1964) reported that extracellular fluid volume (ECFV) was correlated with degree of terrestriality. Hillman (1980) has shown that the more dehydration tolerant species have larger blood volumes than the less tolerant aquatic forms. The amphibian lymphatic system is also unique. Capacious subcutaneous lymph spaces (sacs) are present and two pair of functional lymph hearts persist in adults (see Kampmeier (1959) for a description of lymph heart phylogeny). Carter (1979) has proposed that in aquatic situations these structures provide a pathway for water influx that by-passes the general circulation.

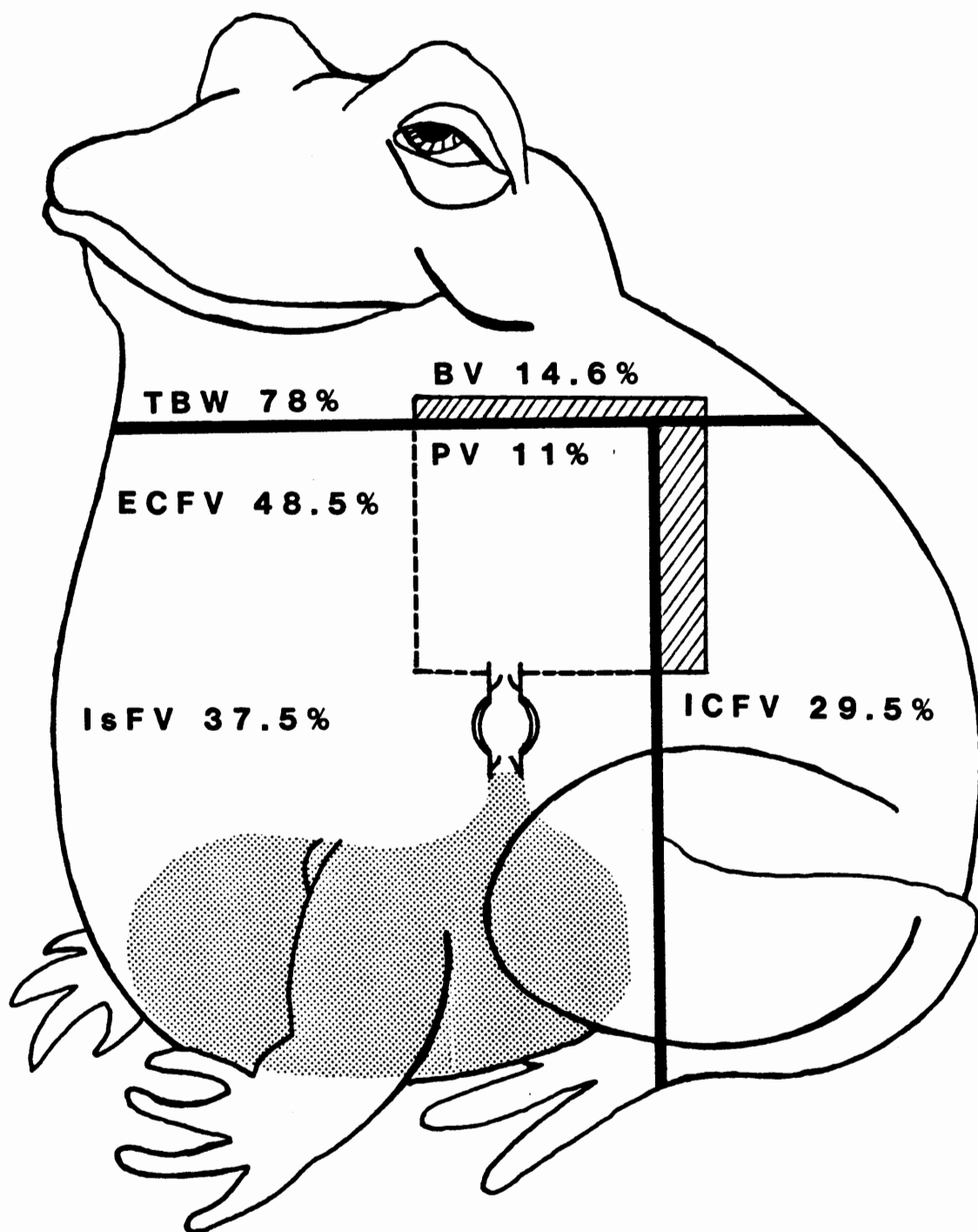
This thesis reports on aspects of lymph heart function that may be adaptive in terrestrial situations. Bufo marinus was used as the model in this study. This fully terrestrial toad has been widely studied with respect to its normal fluid compartment physiology and is a particularly suitable laboratory model because of its large size.

The normal partitioning of body water in B. marinus is depicted in Figure 1. The values given are from Shoemaker (1964). As shown, the total body water (TBW) is partitioned into intracellular (ICF) and extracellular (ECF) fluid compartments. The ECF compartment is divided into interstitial fluid (IsFV) and plasma (PV) volumes. Shoemaker (1964) has shown that two major constituents of the ICF, skeletal muscle and liver tissue, maintain their hydrational status during dehydration in B. marinus. In addition, erythrocytes volume regulate when faced with a hyperosmotic stress (Zygmunt, 1984). Fluid exchange between the ICF and ECF compartments seems unlikely to have an important effect on the acute responses to blood loss reported here and will not, therefore, be considered further.

The vascular space is composed of three sub- compartments. The hatched area in Figure 1 represents the cell fraction of whole blood. A hematocrit (Hct) of 20% is shown. The cellular fraction is divided into solid and fluid components. The extracellular component of blood is the plasma and, as indicated by the dashed lines, is readily exchangeable with the interstitial fluid.

The forces promoting fluid flux at the capillary wall are described as Starling forces and their balance normally results in net filtration. Guyton (1984) provides a detailed description of these

Figure 1. Body fluid compartmentation in Bufo marinus. Data after Shoemaker (1964). TBW = Total body water, BV = Blood volume, ECFV = Extracellular fluid volume, PV = Plasma volume, IsFV = Interstitial fluid volume and ICFV = Intracellular fluid volume.



forces and their normal balance in humans. Plasma filtration into the interstitial fluid compartment results from the combined action of intravascular hydrostatic pressure, negative interstitial fluid pressure and the colloid osmotic pressure (COP) of tissue fluid.

Return of plasma filtrate to the vascular space occurs both at the level of the capillaries and the lymphatic system. The relatively higher COP of plasma tends to draw fluid into the vascular compartment; while, excess water and protein are collected in the tissues by the lymphatic vessels and returned to the low pressure, i.e., venous, side of the vascular circuit. In the amphibia these fluid spaces communicate via the lymph hearts which are depicted in Figure 1 as simple tube hearts protected from retrograde flow by one way valves. That portion of the total extracellular fluid that can be mobilized to augment vascular volume during acute hypovolemic stress is represented by the stippled area in Figure 1. No value is given for the volume of this space.

This model assumes an animal that is fully hydrated, with an empty bladder, under conditions of negligible evaporative water loss. (This is an ecologically atypical situation for these animals, as they would seldom be fully hydrated away from water with an empty bladder.) To investigate the capacity for fluid exchange between the plasma and interstitial fluid spaces, however, it is necessary that ancillary avenues for fluid exchange be eliminated.

Proportional decreases in all fluid compartments would be predicted as a result of evaporative water loss, if water freely equilibrated between the fluid compartments of terrestrial amphibians.

Maintenance of blood volume, in particular, within normal physiologic limits is important for a variety of reasons. Total blood oxygen content and cardiac output are both volume limited. Blood pressure, an important factor in normal fluid exchange, is critically volume dependent.

Shoemaker(1964) reports that when deprived of water, B. marinus exhibit an initial decrease in plasma (hence blood) volume but that this volume stabilizes over water deficits between 5 and 20 %. Unpublished data from our own lab indicates that Hcts are maintained below predicted levels for water deficits up to 20%. The adaptive value of such a response for a terrestrial amphibian faced with ephemeral water supplies is apparent.

Teleologically, it seems reasonable that terrestrial amphibians would defend their blood volume during periods of dehydration. A variety of evidence suggests that mechanisms exist which could do this. However, there is little unambiguous data available to support quantitative assessment of the adaptive value of such a response. Even in the more restricted case of compensation for hemorrhagic loss of blood, there is no clear quantitative understanding of the role, if any, played by the lymph hearts.

Experiments were done to better quantify the role of the lymph hearts during an acute challenge to fluid compartment homeostasis. First, the magnitude of the compensatory response to hypovolemia was investigated in intact animals. Then, similar experiments were done using animals with destroyed lymph hearts. The difference in response between the two treatments should estimate the contribution of the lymph

hearts to fluid mobilization in the resting, hydrated toad. Additional experiments were done on lymph heart destroyed animals to investigate some variables relevant to the interpretation of these primary data.

CHAPTER I

THE RESPONSE TO HYPOVOLEMIA

INTRODUCTION

A rapid compensatory response to hemorrhage induced hypovolemia has been demonstrated in members of all terrestrial vertebrate classes (see, Middler et al. (1968), Lillywhite & Smith (1981)). In addition to other acute cardiovascular responses, evidence suggests that lymphatic fluid is mobilized to augment reduced vascular volumes. This shift of lymph between extravascular and vascular compartments could occur either across the capillary walls or via enhanced lymphatic drainage of the tissues.

Transcapillary filling promoted by changes in the Starling balance of forces at the capillary wall has great potential to move large volumes of fluid rapidly. This is due, in part, to the large surface area for exchange and the proximity of the extravascular lymph reservoir to the vascular space across the capillary wall. Enhanced lymph heart activity would also increase lymph flow, to the extent that lymph is returned to the circulation by way of the lymph hearts.

Middler et al. (1968) have reported an observable change in frequency of lymph heart beat following experimentally induced hypovolemia in toads. Foglia & Braun-Menendez (1941) have shown variation in heart beat frequency in adrenalectomized and hypophysectomized animals and with variation in temperature. These neurogenic organs possess a normally irregular, contralaterally asynchronous beat which, in the case of the posterior pair, can be directly observed through the skin.

Middler et al. (1968) attempted to estimate the response to acute hypovolemia in Bufo marinus. Hemodilution to 58% of prehemorrhage Hct's was observed in intact animals following hemorrhage. Although a second marker (I^{131} radio-iodinated serum albumin) was used in some studies, the authors did not make use of it to determine absolute fluid volumes and volume shifts. A similar response, however, was seen in animals with their lymph hearts destroyed leading the authors to conclude that lymph hearts are not uniquely responsible for the response. Thus, while the lymph hearts seem to represent a potential pathway for lymph return, other acute mechanisms may account for the observed hypovolemic compensation.

In the study reported here, volume compensation following hemorrhage was measured in intact animals using two markers for the plasma space. Use of an injected dye allows for determination of absolute volumes; while, simultaneous analysis of changes in concentration of the injected dye and the endogenous erythrocyte provides a check against fluctuations in the concentration of either marker that may arise from non-compensatory factors. Experience with B.

marinus has shown that considerable changes in Hct occur as a result of activity. These changes, which may exceed 25%, rapidly abate upon cessation of activity. Similar responses are known in other vertebrate groups (Lillywhite & Smits, 1984).

MATERIALS AND METHODS

Animals

Adult Bufo marinus (n = 42) obtained from commercial suppliers were used in this study. Animals were maintained unfed with free access to water prior to experimentation. Both male and female subjects (mass 158g to 366g) were examined. No attempt was made to determine sexual differences in the responses tested.

All animals were fitted with a PE-90 cannula (dead space = 0.1 ml) in the ventral abdominal vein and allowed to recover in a clean plastic container with free access to water for 24 hours prior to experimentation. Cannulas were kept filled with heparinized saline between samplings to prevent clotting. Masses were recorded following evacuation of animals bladders before each experiment.

Determination of fluid volumes

Evan's blue dye and thiocyanate ions (SCN^-) were used to measure plasma volume and extracellular fluid volume, respectively. Standard dye dilution techniques, as described in Shoemaker (1964) or Lillywhite & Smith (1981), were modified as follows to meet the requirements of this study. 300 μL of a 1:3 dilution of 10% Evan's blue was injected through the cannula immediately following collection of a control blood

sample. Two hundred μL of 20% NaSCN was administered as either the Evan's blue diluent or separately following injection of the Evan's blue dye.

Evan's blue standards were prepared by adding appropriate volumes of the Evan's blue solution to 2 mL of 3% BSA. Twenty μL of each standard and the plasma samples were added to 1.0 mL distilled water and the optical density (O.D.) determined at 600 nm on a dual beam spectrophotometer.

Thiocyanate standards were prepared by adding appropriate volumes of the SCN^- solution to 5.0 mL 0.9% NaCl. Forty μL of each sample was pipetted into 1.4 mL 10% TCA to precipitate plasma proteins and protein-bound Evan's blue dye. Standards were similarly acidified, although no protein or dye was present. After centrifugation of samples, 1.0 mL 5% FeNO in 2.5% HNO was added to 1.0 mL of supernatant from each sample and standard and the O.D. determined at 480 nm on a dual beam spectrophotometer.

Plasma volume (PV_{init}) was calculated by dividing the volume of Evan's blue dye injected by the concentration of dye determined from the intercept of a semilog plot of pre-hemorrhage sample concentrations. Initial blood volume (BV_{init}) was calculated from the plasma volume by equation 1 using the mean of the prehemorrhage Hcts (mean control hematocrit, MCHCT)

$$\text{BV}_{\text{init}} = \text{PV}_{\text{init}} / (1 - \text{MCHCT} / 100). \quad (1)$$

Extracellular fluid volume was calculated by dividing the volume of SCN^- injected by the concentration of SCN^- obtained from a sample taken after an equilibration period of 60 min.

Sampling

In order to depict the time course for changes in plasma volume using a dye which is gradually lost from the plasma space, it is first necessary to establish an initial rate of loss for the dye. This was done by taking 5 post-injection samples at 15 minute intervals (see below for individual sample protocol.) After collection of the fifth sample the cannula was allowed to drain into a heparinized 10 ml syringe until a volume of blood equal to 2.0% of the animal's body mass had been removed. This blood was retained for reinfusion after completion of the experiment.

Following hemorrhage, the acute phase of compensation was surveyed. Pilot studies indicated that post-hemorrhage recovery would be rapid, therefore, as many samples as possible were taken during this period. Five post-hemorrhage samples were collected at 5 min intervals. An additional 4 samples were taken at 15 min intervals to reestablish the baseline rate of dye loss following compensation. Including the control sample, 15 samples were collected in all.

In order to assure that samples obtained were representative of the general circulation and not peculiar to the local environment of the cannula, the following protocol was used to obtain all samples. Saline filling the cannula was allowed to drain off and an empty syringe barrel was fitted to the cannula. The syringe was allowed to fill with 0.1 to

0.2 ml of blood and removed. Two heparinized micro-hematocrit tubes were filled with approximately 40 μ l of blood each. The previously drawn blood was then reinfused and the cannula flushed with 0.1 ml of heparinized saline. Hematocrits were determined after centrifugation for 5 minutes and plasma samples were refrigerated for colorimetric analysis, as previously described.

Animals were sampled unanaesthetized. To avoid complications due to extemporaneous activity caused by intermittent handling during sampling each animal was kept in a plastic container placed over a grate through which the cannula was accessible. It was desired that the animals be tested at rest, as Hct was found to fluctuate with activity. However, B. marinus are prone to struggle when confined or restrained and periodically would engage in vigorous activity bouts.

Determination of Blood Volumes During Compensation

Plasma and blood volumes following hemorrhage (PV_i & BV_i) were determined by subtracting the volume hemorrhaged (VH) and ($VH * (1 - Hct_H / 100)$) from the initial blood and plasma volumes, respectively. Here, Hct_H is the Hct at the time of hemorrhage.

Plasma and blood volumes (PV_i & BV_i) following hemorrhage were calculated from observed changes in the concentration of Evan's blue and Hct using equations 2 and 3.

$$PV_i = PV_{init} * \frac{C_{Hc}}{C_i} \quad (2)$$

$$BV_i = BV_{init} * \frac{(Hct_i)}{(Hct_H)} * CV - \frac{VH * Hct_H}{CV} \quad (3)$$

where:

- C_{Hc} = concentration of dye at hemorrhage corrected by extrapolation of pre-hemorrhage concentrations to t_i
- C_i = dye concentration at t_i
- Hct_H = hematocrit at hemorrhage
- CV = volume of cells initially present
- VH = volume of blood hemorrhaged
- Hct_i = hematocrit at t_i
- t_i = time sample was taken

The term C_{Hc} in the numerator of equation 2 corrects for the continued loss of dye during the period of compensation, assuming a rate of dye loss equal to the pre-hemorrhage rate. This is a conservative assumption as a decrease in blood pressure following hemorrhage would likely result in a concomitant decrease in filtration of dye.

The cell volume remaining after hemorrhage (CV_H) was calculated by equation 4 as

$$CV_H = (BV_i - VH) * Hct_H. \quad (4)$$

This volume of cells is used in equation 5 to calculate post hemorrhage blood volumes (BV) from the plasma volumes determined from the Evan's blue washout.

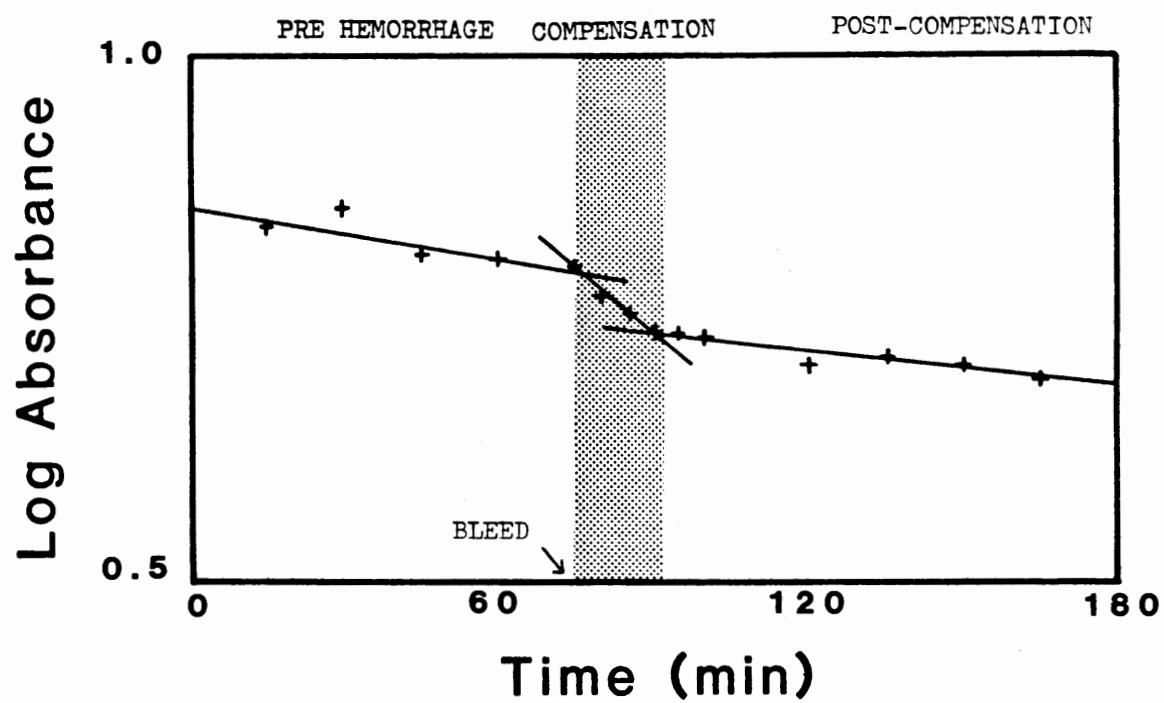
$$BV = PV + CV \quad (5)$$

Statistical Methods and Data Analysis

The fundamental analysis of washout curves for the experiments described in Chapters 1 and 2 was done as follows. Each experimental trial was analysed for time trends in the data. The intervals subtended by these trends are depicted in Figure 2. The sample taken just before hemorrhage was considered to be the last element of the pre-hemorrhage interval and the first element of the putative compensation interval. The intersection of the compensation and post-compensation intervals was determined using the multiple regression method described by Draper and Smith (1981) in their discussion of analysis of time trends in data. Actual analysis was done using the Multiple Regression: Subprogram Regression of Statistical Package for the Social Sciences (SPSS) by Nie et al. (1975). Analysis was done using semi-log plots of raw absorbance data. All reasonable groupings of the data were analysed and the compensation interval was taken to end at the sample following the predicted intersection point of the model having the smallest residual sum of squares.

Testing for significance of differences between the slopes of lines within the intervals so determined was done using the analysis of variance method for tests of more than 2 slopes described by Zar (1984). Time to recovery (TTR) was considered to be the time of the compensation

Figure 2. Example of raw data from hypovolemia experiment using an intact animal.



interval; therefore, TTR for individual animals is a multiple of the 5 min sampling intervals used.

RESULTS

Fluid volume determinations for the 8 animals used in this study are shown in Table 1. The degree of hypovolemic stress was calculated individually for each animal as the ratio of blood volume removed (VH) to BV_{init} .

If compensation were to occur during the time course of these experiments, 3 time trends should appear in the dye concentration data. Between the times of dye injection and hemorrhage a slow exponential decrease in dye concentration should accompany the unavoidable loss of dye from the vascular space (see Figure 2, pre-hemorrhage.)

Following hemorrhage, concentration should decline precipitously as unlabeled fluid fills the vascular space to compensate for the deficit (see Figure 2, compensation.) Acute compensation can be inferred if slopes of the first and second time trends are significantly different. The magnitude of the response can be estimated from the divergence of these two lines during the period of compensation. Compensation has ended when the decline in concentration brought about by the hemorrhage has ceased (see Figure 2, post-compensation.) The expected contours in the absorbance data were observed in all control animals tested. In all cases, the slope of the compensation time trend was significantly different from the others.

The profiles for Evan's blue concentration and Hct are shown in Figure 3. As can be seen, both decline precipitously following

TABLE I

FLUID VOLUME SUMMARY FOR INTACT ANIMALS (n = 3, except ECFV n = 3)

	Initial Plasma Volume (ml/100g)	Hematocrit	Initial Blood Volume (ml/100g)	Stress (...)	Time To Recover (min)	Recovery Volume (...) E.B. Hct	Recovery Rate (ml/min) E.B. Hct	Extracellular Fluid Volume (ml/100g)
Mean	7.32	21	9.25	22.3	17.5	96 107	.21 .33	49.9
S.E.	.56	2.9	.52	1.2	1.3	3.5 3	.05 .06	5.9
Range	5.24 - 10.36	13 - 35	7.66 - 11.92	16.6-24.9	15 - 25	87-110 97-116	.07-.47 .12-.67	41.6 - 61.4

hemorrhage, with Evan's blue concentration attaining constancy after about 15 min post hemorrhage. Means of the Hct determined values in Figure 3 fall within 2 SE of the mean dye determined values. It can be seen, however, that Hct as a % of control continues to decline over the full 25 mins shown and that 2 SE of the Hct determinations fail to capture the mean of the dye values at 20 and 25 min post-hemorrhage. Hemodilution exceeds Evan's blue washout at every interval.

Blood volume recovery data is shown in Figure 4. This figure is an algebraic variant of Figure 3 and as expected depicts similar relationships between estimates derived using the two markers. It can be seen that compensation as inferred from the dye washout is complete 15 min post hemorrhage; while the profile of Hct determined volumes would suggest that compensation continued through 25 min.

These experimental results are also summarized in Table 1. The percent recovery shown in Figure 4 is lower than that given in Table 1 because variation in individual TTR's have not been corrected for in the abscissa. The time to recovery reported is the arithmetic mean of individually determined recovery times measured as multiples of the five minute sample intervals. Of the 8 individuals tested, 5 recovered in 15 min, 2 in 20 min and 1 only after 25 min.

Percent recovery volume is the mean ratio of the recovery blood volume at TTR and BV_{init} for each individual. Percent recovery is given using volume estimates based on the washout of both markers. The values are not significantly different. The recovery rates shown are means of slopes obtained from individual blood volume profiles during the interval of compensation.

Figure 3. Percent change in Evan's blue concentration (EB) and hematocrit (Hct) following hemorrhage in intact animals. Mean \pm 2 SE, (n = 8).

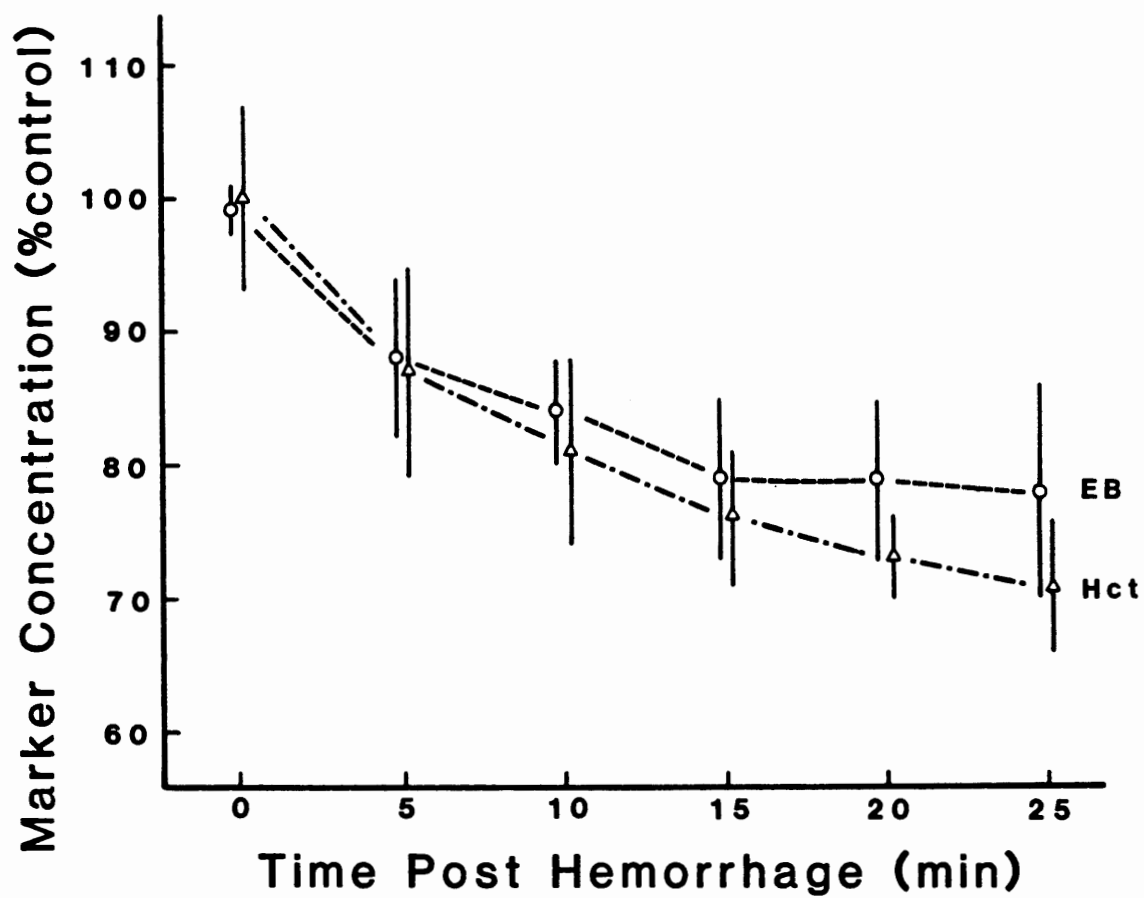
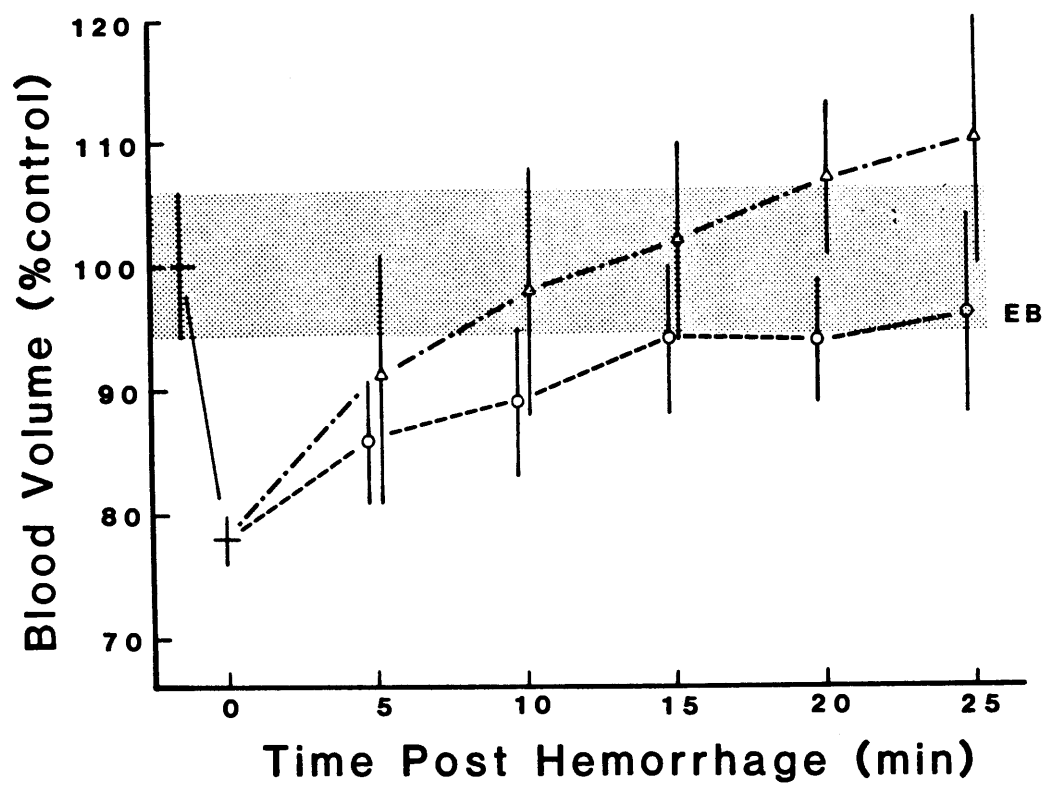


Figure 4. Blood volume as a percent of control in intact animals.
Mean \pm 2 SE, (n = 8). Values determined from
Evan's blue (EB) and hematocrit (Hct).
Error in BV0 stippled (\pm 2 SE).



The effect of hemorrhage on the distribution of SCN^- is shown in Figure 5. Following an equilibration period of about 45 min the decline in the log transformed absorbance is linear. No perturbations of this linearity can be attributed to the hemorrhage or concomitant compensation. Determination of absolute ECFV was not done on this data. The value for ECFV given in Table 1 is from an independent determination done on 3 toads. One of these toads provided data that has been incorporated into this study. The other 2 are included in the study which follows.

DISCUSSION

Published values for plasma volume determined by dye dilution using Evan's blue dye vary considerably. Thorson (1964) provides a plasma volume estimate of 4.7ml / 100g for Bufo marinus, whereas, Shoemaker (1964) reported mass specific plasma volumes more than double this amount for the same species (11.1ml / 100g). The value determined in this study (7.3ml / 100g) agrees closely with the value given by Prosser & Weinstein (1950) that is commonly used as an estimate (7.0ml / 100g).

In order for dye dilution estimates of fluid volumes to be accurate it is necessary for the concentration of dye in the samples taken to equal the mean concentration of the space being measured (Lawson, 1962). Thorson makes the claim that circulatory mixing was complete over the sampling period used; however, the dramatically lower values reported suggest a sampling period longer than 30 min post injection may be desirable.

Figure 5. Distribution of thiocyanate ions during acute hypovolemia in individual intact animals.

The coefficient of variation of the blood volume estimate (15.9%) is lower than for the plasma volume estimates (21.6%). Variation in Hct and plasma volume appear to be coupled, indicating that the controlled variable is blood volume. Animals with lower red cell volumes have correspondingly higher plasma volumes and vice versa.

The discrepancy between the estimates of compensation based on dye washout versus hemodilution probably arise from 2 sources. First, the assumption that dye filtration following hemorrhage proceeds at a rate consistent with that observed before bleeding probably leads to an underestimate of dye washout. Perhaps more significant, however, may be the return of label to the circulation over time as dye accumulates in the lymph.

These results are comparable to those obtained by Middler et al. (1968). Hct washout to 58% of control followed hemorrhagic stress of approximately 50% (3.5mL/100g) in their study. In the study reported here a mean hemorrhagic stress of 22.3% produced a washout of 71%. The Middler study does not provide sufficient data to justify a comparison of the TTR.

The rate and precision of blood volume regulation following acute blood loss determined in this study are remarkable, although not entirely unexpected. As previously described, changes in Hct following activity of 27% to 36% have been seen among toads in our lab. The activity induced hemoconcentration is reduced to 25% after 20 - 30 min rest. This would suggest that a volume deficit comensurable with the observed increase in Hct has been replaced in that period of time.

CHAPTER II

THE EFFECT OF LYMPH HEART DESTRUCTION

INTRODUCTION

A variety of studies have suggested that the lymph hearts are vital to the maintenance of normal fluid compartment physiology. Foglia (1939, 1941) has shown that fatal hemoconcentration and edema follows the loss of functional lymph hearts in Bufo aboretum, indicating net transfer of fluid from vascular to extravascular spaces. This result was observed whether the hearts were destroyed by electrocautery, in which case the lymph channel was destroyed, or were simply denervated, in which case only the beating of the heart was arrested. This led Foglia (1941) to characterize the hearts as suction pumps.

The rate of lymph formation and the capacity of the lymph hearts to return this fluid to the circulation have been the subject of many investigations. Ito (1926) reported hemoconcentration of 5 - 46% in 10 mins following lymph heart destruction. If this change results solely from stoppage of lymph flow, an animal with a 7% plasma volume must be cycling a volume of lymph equal to 5 times its body mass per day.

Conklin (1930) attempted to verify these results after destruction of the posterior hearts alone. She was able to manipulate a subset of her data to produce agreement with the results of Ito. Ten years later, however, Foglia (1939) was able to show that a single functional lymph heart is sufficient to forestall the fatal consequences of lymph heart destruction and presumably acute hemoconcentration as well.

Two conclusions are implicit in these studies. First, that volume of fluid, which lost produces the observed hemoconcentration, must normally be returned to the circulation. Second, all this fluid is returned via the lymph hearts as the volume lost is the result of net capillary filtration.

In 1968, Middler et al. took the opposite approach. Hemodilution following acute hypovolemia was determined both in intact animals and animals without lymph hearts. Intact animals showed Hct reduction to 84% and 47% of control; however, lymph heart destroyed animals hemodiluted to 37% of initial Hct. The observation that lymph heart contraction rate increased following hemorrhage in the intact animals suggests that the lymph hearts contribute to compensation. The magnitude of this contribution is the difference in hemodilution between the two treatments.

The minor contribution of the lymph hearts to hypovolemic compensation suggested by the Middler study seems to contradict the fundamental importance of these organs in maintenance of fluid balance implied by the studies of Ito (1926), Conklin (1930), Foglia (1939; 1941) and Zwemer & Foglia (1943). Bashir (1967) has determined that the

lymph hearts are vital for the return of lymph to the circulation in Rana catesbeiana. This discrepancy deserves further study. The method of the previous section is here used to measure the differences in compensation between animals with and without lymph hearts.

MATERIALS AND METHODS

Surgical Destruction of the Lymph Hearts

Destruction of lymph hearts was done by the method of electrocautery as described in Foglia (1939). In the discussion that follows animals without lymph hearts are frequently designated LH- and intact animals as LH+. Animals were anaesthetized for surgery using MS-222. Because the lymph hearts lie imbedded in connective tissue, the persistence of the heart beat during the surgery is a valuable visual aid to identify the heart during surgery. Therefore, animals were removed from the anaesthesia as soon as the corneal reflex was lost since longer periods of exposure were found to arrest the lymph heart beat.

The anterior lymph hearts lie between the suprascapula dorsally and the transverse process of the 3rd vertebrae ventrally. They were exposed by making a 1.5cm incision parallel to the vertebral margin of the suprascapula and separating the rhomboides muscle from the musculature of the vertebral column. Heart beat was usually not visible in the anterior hearts. In an effort to assure complete destruction, all connective tissue in the exposed cavity was cauterized.

The beating of the posterior hearts was readily observable beneath the overlying skin just lateral to the caudal end of the ilium. A 1.5 - 2.0cm incision was made through the skin over the region where the

pulse was observed. To minimize the burning required to fully destroy the hearts, excess connective tissue was cut away prior to cauterizing these structures.

Where hearts were observed beating, application of the current caused an immediate cessation of the beat; therefore, the location of each heart was carefully observed before thorough cautery of the area. All incisions were closed with 3 - 5 single silk sutures. Loss of lymph through the sutured incisions was minimal provided the animals remained in an upright posture.

Where sham operations were performed they consisted of making incisions in the skin and suturing in the normal manner. No cautery of any tissues was done on these animals.

Following surgery, each animal had its bladder drained and was weighed. Animals were placed in dry plastic containers to recover. A moist paper towel was placed over the container to retard evaporative water loss during recovery. Following 3 hrs recovery the animals appeared to behave normally.

Sampling of Arterial and Venous Hematocrits

Initial data collected from animals with destroyed lymph hearts suggested that post-hemorrhage venous Hcts may have been biased by changes in perfusion through the vascular circuits being sampled. Decreases in blood flow rate (BFR) would tend to promote red blood cell (RBC) pooling, resulting in sample Hcts above mean circulatory Hct.

Five animals were outfitted with ventral abdominal vein and systemic arch cannulas. Each animal was tested separately following a

sham operation and a day later actual lymph heart cauterization. Several pre-hemorrhage samples were collected to compare control A/V Hcts. Following hemorrhage of 2ml/100g mass, additional samples were collected at 5-10 min intervals for 30 min to determine the effect of hemorrhage on Hcts taken from these circuits.

Absolute volume determinations were not done. The animals were kept unconfined in dry plastic containers covered with moist paper towels during the course of these experiments.

Total Plasma Protein

Plasma samples were taken from a group of 6 lymph heart destroyed animals every 3 to 6 hours until death. Two of the animals were similarly sampled for 1 day prior to the lymph heart surgery, following a sham operation. Plasma samples were collected as previously described. Lymph samples were obtained, at the same time, through a small incision in a dorsal lymph sac. Hcts were taken and total protein determined using the biuret method (Sigma Technical Bulletin, Total Protein kit #540).

RESULTS

Determinations of the initial fluid volumes for the LH- animals used in the hypovolemia experiments are summarized in Table 2. Mean plasma and blood volumes for this group (6.07 ± 0.96 and 7.79 ± 1.04 , Mean \pm 2 SE) are significantly less (t-test, $P < 0.05$) than for intact animals (7.32 ± 1.12 and 9.52 ± 1.04 , Mean \pm 2 SE). Hematocrits were higher (24 LH-, 21 LH+) but not

TABLE II

FLUID VOLUME SUMMARY FOR ANIMALS WITH LYMPH HEARTS DESTROYED (n = 6)

	Initial Plasma Volume (ml/100g)	Hematocrit	Initial Blood Volume (ml/100g)	Stress	Recovery Volume (%) E.B. Hct	Recovery Rate (ml/min) E.B. Hct
Mean	6.07	24	7.79	25.0	77 61	.02 - .15
S.E.	.48	2.4	.54	1.6	3.5 5.0	.015 .04
Range	4.61 - 7.70	16 - 32	6.49 - 9.71	20.1-30.3	68 - 88 49 - 83	-.03 - - .27 - .06 -.04

significantly so. The degree of hemorrhagic stress in this group was correspondingly higher (25.0%) than for intact animals (23.2%).

Evan's blue absorbances were analysed for time trends in the data using methodology described in Zar (1984). The putative compensation interval for this analysis was taken to be 20 mins post hemorrhage. This includes the sample following the mean time to recovery observed for intact animals. No significant differences (ANOVA, $P < 0.01$) were found in slopes of the 3 intervals for 6 of the nine animals tested. Further analysis indicated no substantive difference between the 3 exceptional toads and the control animals; therefore, these surgeries were considered to have failed and data taken from these animals was excluded from analysis.

The relative changes in Evan's blue concentration and Hct are shown in Figure 6. Evan's blue concentration following hemorrhage remained constant while Hcts rose 28%. Blood volume profiles constructed from both markers are shown in Figure 7. As would be predicted from the results in Figure 6, volumes determined from Hct washout are lower for all intervals, indicating continuing loss of plasma during the compensation interval. Percent compensation in the intact and lymph heart destroyed animals is compared in Figures 8 and 9 for the dye and Hct markers, respectively. The ordinate in these figures was determined by dividing the volume returned to the circulation by the hemorrhagic stress.

Figure 6. Percent change in Evan's blue concentration (EB) and hematocrit (Hct) following hemorrhage in animals with lymph hearts destroyed. Mean \pm 2 SE, (n = 6).

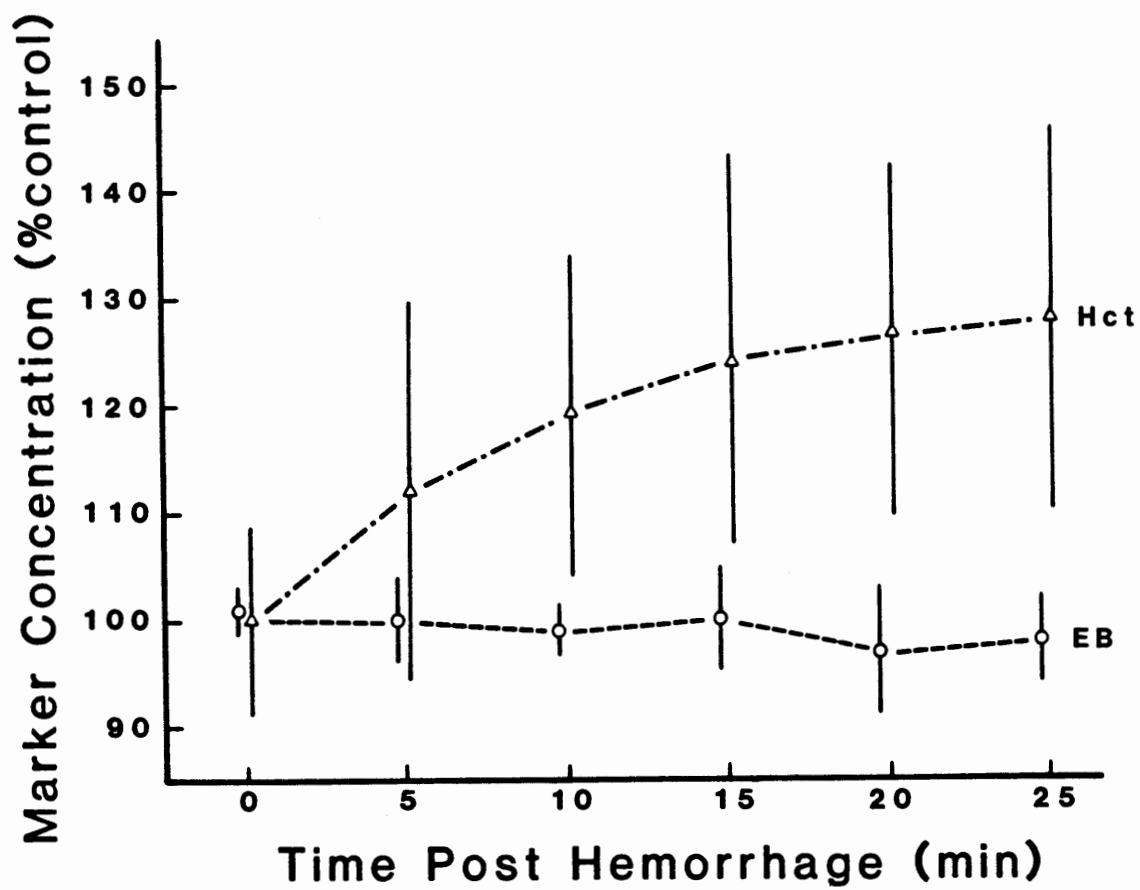


Figure 7. Blood volume as a percent of control in animals with lymph hearts destroyed. Mean \pm 2 SE, (n = 6). Values determined from Evan's blue (EB) and hematocrit (Hct) Error in BVO stippled (\pm 2 SE).

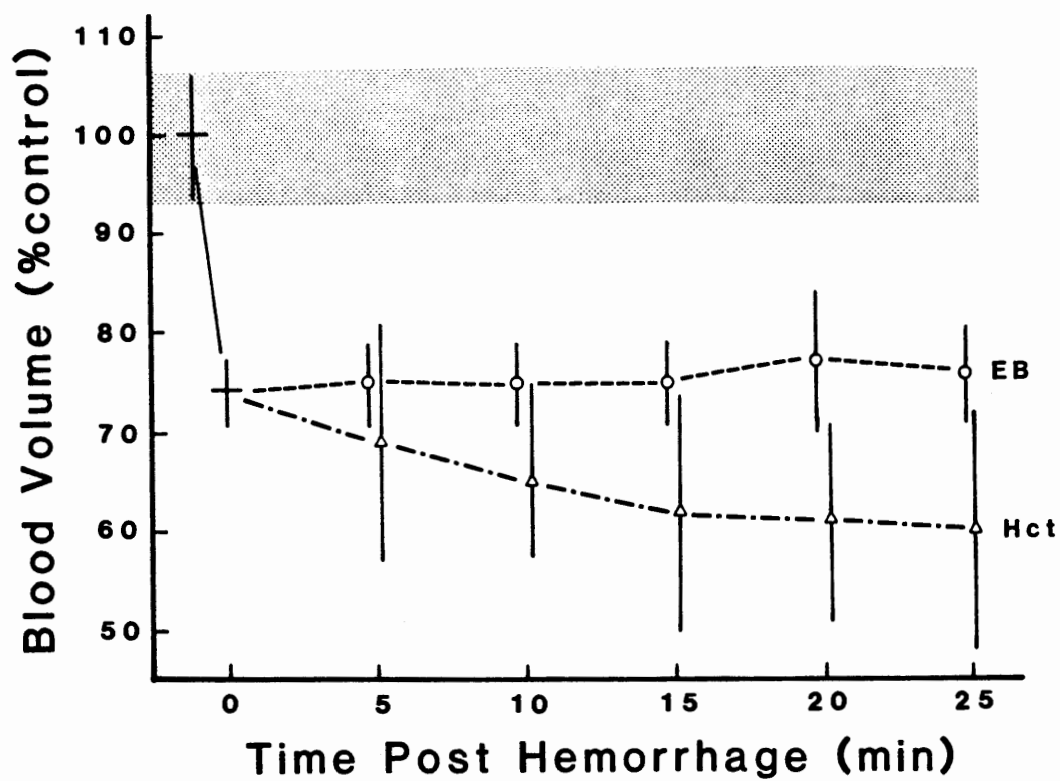


Figure 8. Blood volume recovery as a percent of volume hemorrhaged, determined from Evan's blue washout in intact animals (LH+, n = 8) and animals with lymph hearts destroyed (LH-, n = 6).

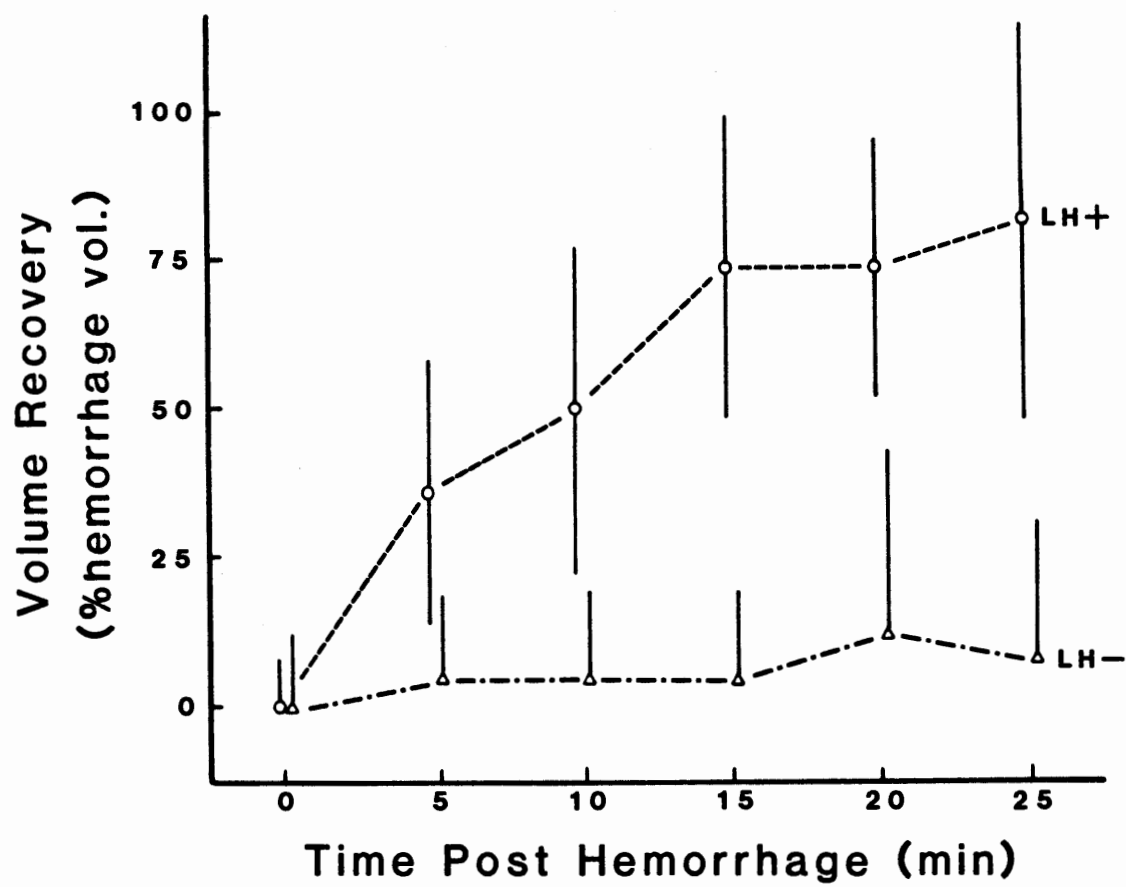
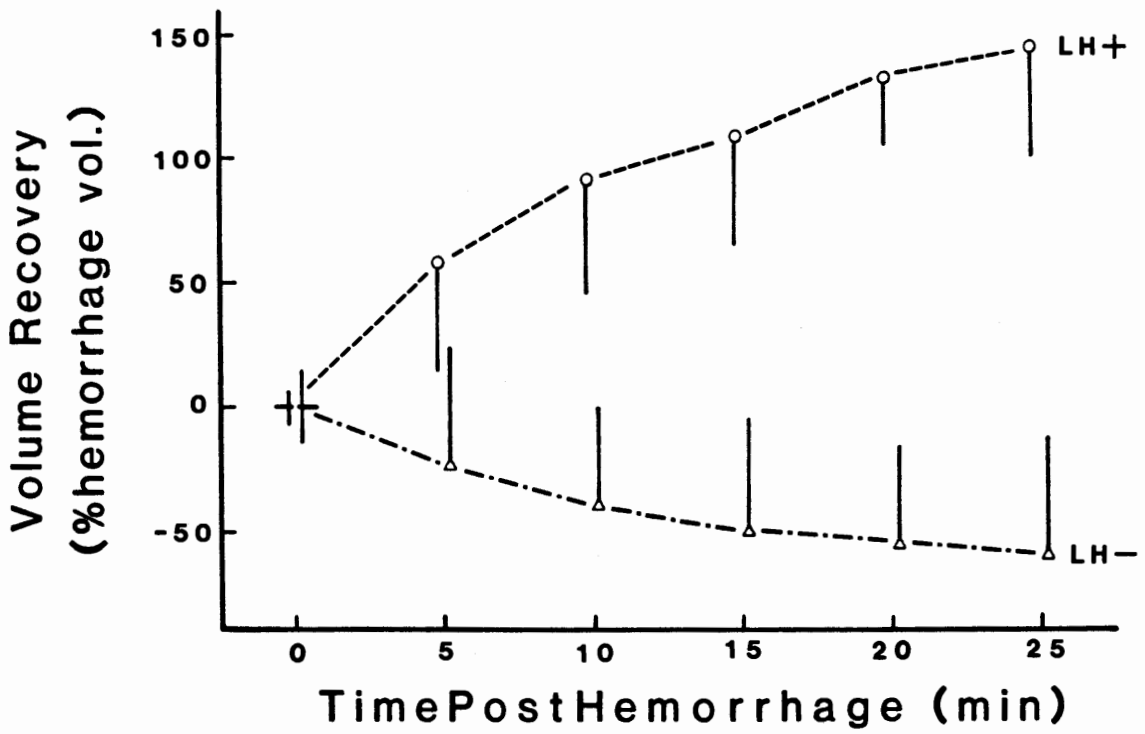


Figure 9. Blood volume recovery as a percent of volume hemorrhaged, determined from hematocrit washout in intact animals (LH+, n = 8) and animals with lymph hearts destroyed (LH-, n = 6). Mean \pm 2 SE.



Arterial vs Venous Hematocrits

Arterial Hcts tended to be 1-2% higher than venous, in both the sham operated and lymph heart destroyed animals. Following hemorrhage in the sham operated animals both arterial and venous Hcts exhibited characteristic declines. Decreases in arterial Hct ranged from 82% to 63% of control and were generally 5% to 10% lower than corresponding venous Hcts. In the animals without lymph hearts, the prehemorrhage Hct profiles paralleled each other as well. Interestingly, the dramatic post hemorrhage increase in venous Hct observed previously was not seen in these animals. Both arterial and venous samples showed minor hemoconcentration.

Chronic Effects of Lymph Heart Destruction

The long term effect of lymph heart destruction on Hct was quite variable. Hematocrits at death ranged from 28 to 65. Terminal Hct appears coupled to initial Hct, generally exhibiting about a two fold increase over the latter.

Protein concentrations in plasma and lymph of the lymph heart destroyed animals and controls do not differ significantly. Analysis of the data failed to reveal any time-linked decrease in protein concentration in either compartment. The ratio of plasma to lymph protein was 2.6 ± 0.38 .

DISCUSSION

The decrease in initial plasma and blood volumes of the lymph heart destroyed animals compared with the intact animals suggests that a

significant loss of plasma occurs over the 3 hour operative and recovery period. The larger hemorrhagic stress reported for this group further testifies to this effect.

Mean recovery volume for the animals with destroyed lymph hearts is only two percent above the mean post hemorrhage volume (Figure 8) and recovery rates reported are 10% of the values determined for intact animals. The concentration and volume profiles in Figures 4 and 5 similarly indicate that the lymph heart destroyed animal is unable to replenish depleted vascular volumes.

In another class, Reptilia, hypovolemic compensation is believed to occur by transcapillary fluid shifts (Smits and Lillywhite, 1985). The findings of Middler et al. (1968) would suggest a similar mechanism is important for B. marinus. The lack of a response by the lymph heart destroyed animals in this study, however, argues strongly that any transcapillary mechanism is of secondary importance. Taken together, the post-operative blood volume decrement in unstressed animals and the inability to compensate for acute hypovolemic stress, indicate that the lymph heart pathway is fundamentally important for lymph return.

The two groups also showed differences with respect to the effects of activity on Hct. Preparation of animals for experimentation, including weighing, bladder evacuation and confinement resulted in a transient erythrocythemia, frequently as large as 40%. This effect abated after 10 to 20 minutes of quiescence in the intact animal. The increases in hematocrit following hemorrhage of the lymph heart destroyed animals (Figure 5) persisted for the 90 min duration of the experiment.

In the control experiments activity bouts were infrequent. In the lymph heart destroyed group, however, cannula flow was typically weak and removal of the animal from confinement in order to hemorrhage was frequently required. The increases in Hct seen in Figure 5 are most likely attributable to activity associated with this handling.

The A/V Hct studies corroborate this interpretation. The animals used in this study were handled through out the sampling procedure. Mean Hcts for this group were higher than for either of the other groups. The absence of post-hemorrhage erythrocythemia in this group indicates that the scope for further increase in Hct has been taken up by the initially elevated Hcts.

The disparity between the dye and red cell markers during this activity is nonetheless paradoxical. Unless the animal is filtering plasma, as opposed to serum, a concomitant increase in plasma protein and hence, protein bound Evan's blue dye should be seen. No evidence of increased plasma protein concentration was observed (see Figure 5.) This is supported by total plasma protein determinations in the study of chronic effects of lymph heart destruction, where no change in plasma protein concentration was observed. However, if plasma is being filtered, lymph protein concentrations should be increasing toward plasma levels which they are not.

In the preceding study, Evan's blue determinations underestimated plasma volume recovery relative to Hct determined values. Here, Evan's blue determinations appear insensitive to increased filtration rates encountered during the experiment. Therefore, it can be concluded that changes in plasma volume are best marked by observing changes in Hct.

The importance of the dye used in this study should not be ignored. Data from the Evan's blue provided the basis for determination of the volume measurements. In addition the analysis of recovery times could not have been done using Hcts measured as whole number percentages. The problems related to interpretation of the Evan's blue data for determining volumes should not compromise the dye washout kinetics analysis used to determine the duration of the acute phase of the compensation response. Clearly these results point to the importance of controlling the physiologic state, e.g., stressed vs non-stressed, active vs non-active, in studies where blood volume is an important parameter.

Survivorship among surgically modified animals averaged 2 days post surgery. This is consistent with the results of Foglia (1939). It was mentioned above that application of current from the cautery electrode caused cessation of the heart beat. The 3 individuals, previously described, that exhibited anomolous post-hemorrhage blood volume profiles also differed from the others with respect to survivorship.

Despite the concerns of Conklin (1930) that the surgery was traumatic for the animal, no adverse behavioral effect were apparrent. Experience from this study would suggest that no reasonable effort should be spared to assure the efficacy of the surgery. In the case of the compensaton reported by the Middler group for lymph heart destroyed animals the possibility that the surgeries weren't successful must be considered. Unfortunately the authors didn't provide any information in this regard.

In response to acute hypovolemic stress of approximately 20% - 25%, intact animals examined here were able to compensate at a rate of about 0.25 ml/min to achieve full recovery in less than 20 min. This response was accomplished by redistribution of extracellular fluid. Cauterization of the lymph hearts blocks this response. No compensation occurred in animals with thoroughly destroyed lymph hearts, suggesting the absence of a transcapillary mechanism for lymph return.

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